In Vivo Effects of mRNA-lipid Adducts and Mitigation Strategies

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Abstract

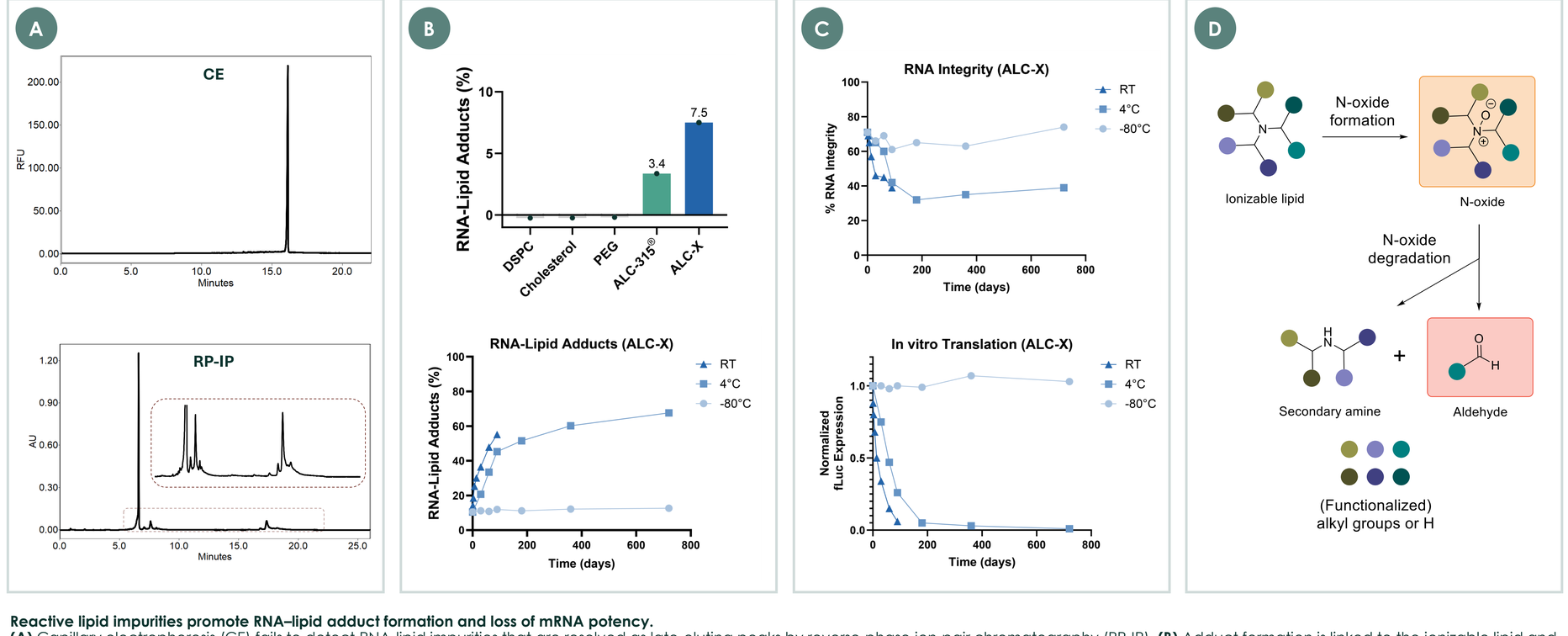
Background and Objectives

Lipid nanoparticles (LNP) have emerged as a leading platform for RNA delivery. However, effective delivery requires stringent control of lipid raw material quality, as reactive impurities in ionizable lipids can chemically modify RNA, forming RNA–lipid adducts that impair translation[1]. Here, we focus on specific lipid impurities associated with adduct formation and demonstrate their impact on *in vivo* protein expression following intravenous administration of mRNA-LNP. We further present multiple mitigation strategies—including adduct-resistant ionizable lipid designs, lipid re-purification, rapid point-of-care formulation process, optimized storage buffers, and lyophilization—to prevent adduct formation and preserve mRNA-LNP potency.

Materials and Methods

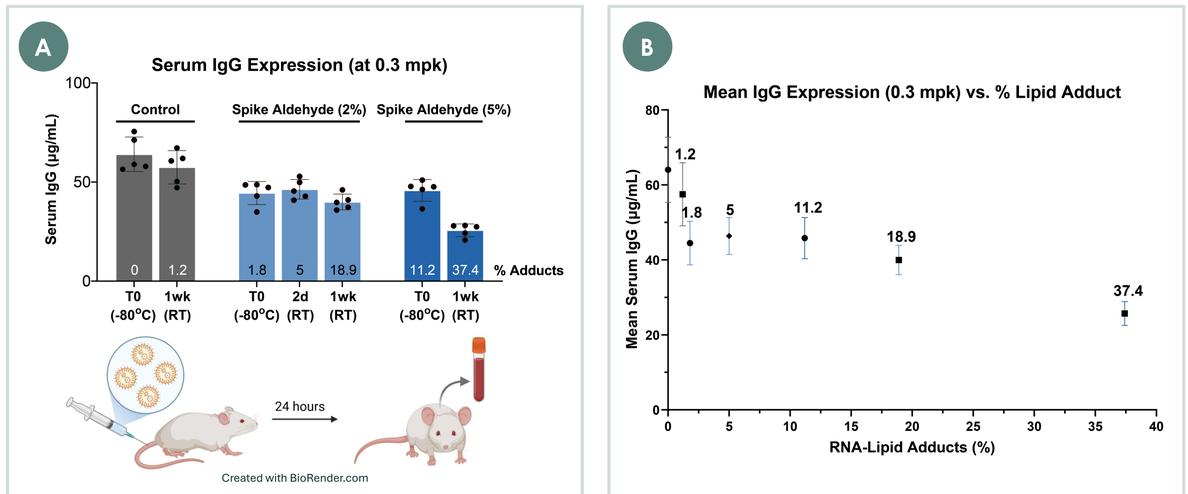
Raw ionizable lipids and LNP were screened for key reactive impurities (aldehydes and N-oxides) by reversed-phase chromatography coupled with mass spectrometry[2]. RNA-lipid adduct formation was induced in binary RNA-lipid mixtures (3:1 buffer:ethanol) or formulated LNP, and quantified using two orthogonal reverse-phase ion-pair chromatography (RP-IP) methods[1,3]. Protein expression was assessed *in vitro* (cell-free wheat germ extract system) and *in vivo* following intravenous administration of mRNA-LNP (0.3 mg/kg, CD1® mice).

1 Reactive Lipid Impurities Drive RNA Adduct Formation and Loss of mRNA Potency



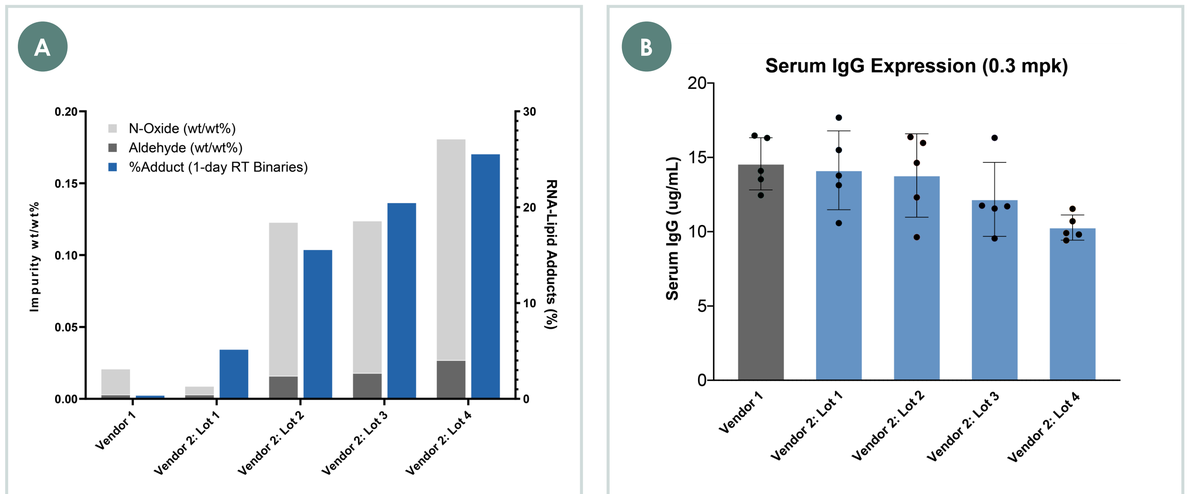
Reactive lipid impurities promote RNA–lipid adduct formation and loss of mRNA potency.
(A) Capillary electrophoresis (CE) fails to detect RNA-lipid impurities that are resolved as late-eluting peaks by reverse-phase ion-pair chromatography (RP-IP). **(B)** Adduct formation is linked to the ionizable lipid and is dependent on time and temperature. **(C)** RNA-lipid adducts, together with RNA integrity, reduce *in vitro* protein expression. **(D)** Proposed mechanism of ionizable lipid degradation: oxidation of the tertiary amine forms an N-oxide intermediate, followed by acid/base-catalyzed hydrolysis to generate a secondary amine and a reactive aldehyde that can reacts with nucleic acids to form RNA–lipid adducts.

2 Spiked Aldehyde Impurities Reduce *in vivo* mRNA-LNP Potency



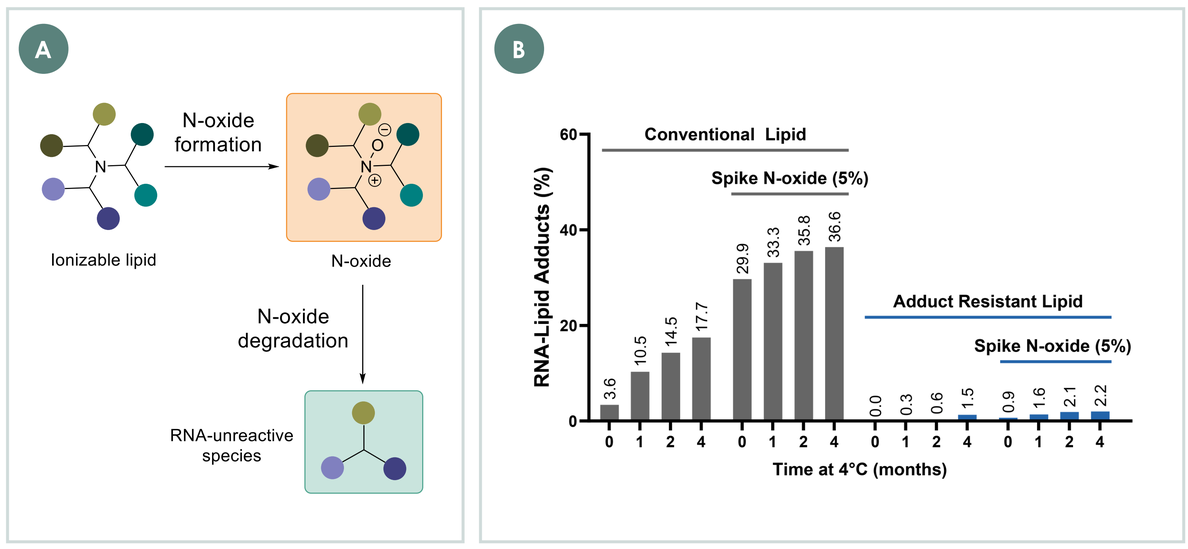
Spiked aldehyde impurities increase RNA–lipid adduct formation and reduce *in vivo* IgG expression.
(A) Serum IgG expression following intravenous administration of mRNA-LNP formulations containing 2% or 5% spiked aldehyde impurity. Formulations were stored at −80°C or room temperature (RT) for the indicated durations. RNA–lipid adduct levels (%) are shown for each group. **(B)** Serum IgG expression as a function of RNA–lipid adduct levels across the indicated formulations.

3 Raw Material Impurities Compromise *in vivo* mRNA-LNP Performance



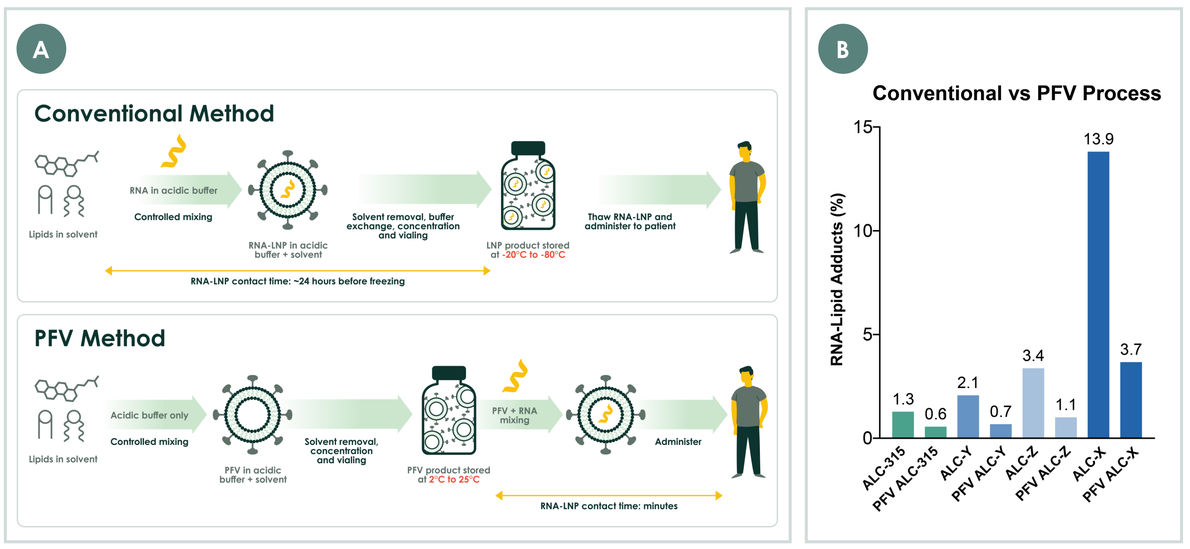
Raw material impurities are associated with RNA–lipid adduct formation and reduced *in vivo* protein expression.
(A) Lipid lots with higher N-oxide and aldehyde impurity levels showed greater RNA–lipid adduct formation in binary RNA-lipid mixtures following 1 day at room temperature (RT). **(B)** Serum IgG expression following intravenous administration (0.3 mg/kg) of mRNA-LNP formulated with the indicated lipid lots.

4 Adduct-Resistant Ionizable Lipid Design Reduces N-Oxide Driven Adduct Formation



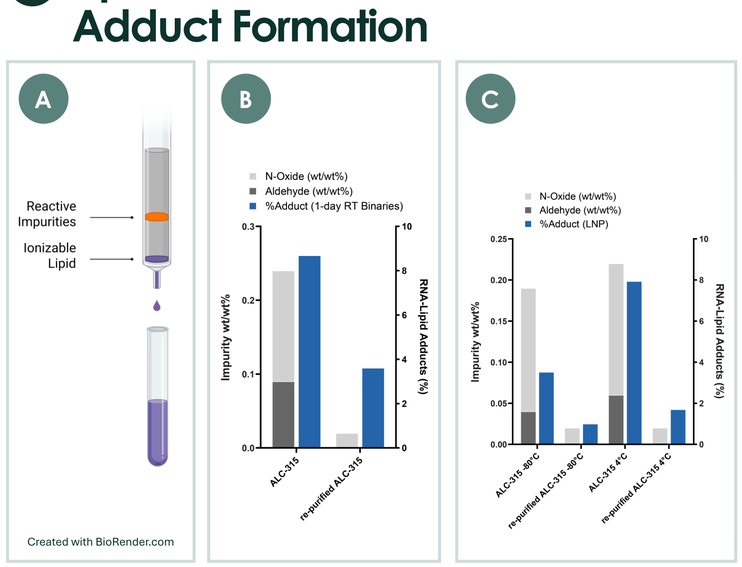
Adduct-resistant ionizable lipids mitigate N-oxide–driven adduct formation during storage.
(A) Proposed degradation pathway in which ionizable lipid N-oxide formation and subsequent degradation generate RNA-unreactive species rather than reactive aldehydes. **(B)** Adduct formation in LNP formulated with conventional or adduct-resistant ionizable lipids spiked with 5% N-oxide impurity and stored at 4°C for up to 4 months.

5 Pre-Formed Vesicle (PFV) Formulation Process Reduces RNA–Lipid Adduct Formation



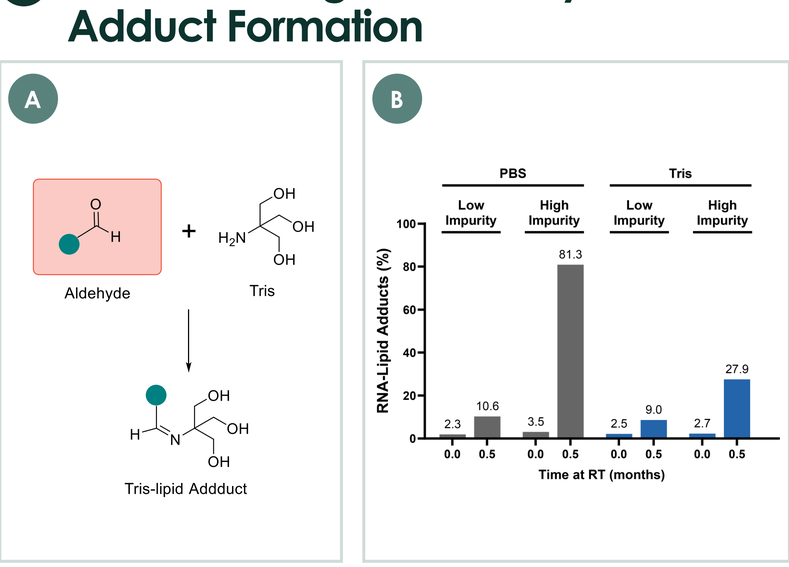
Pre-formed vesicle (PFV) formulation process enables rapid RNA loading while reducing RNA–lipid adduct formation.
(A) Comparison of conventional and PFV formulation processes. Conventional formulations involve direct mixing of RNA with lipids, whereas PFV formulations involve loading RNA into pre-formed vesicles, reducing RNA–lipid contact time from approximately 24 hours to minutes. **(B)** RNA–lipid adduct levels in ALC-315®, ALC-Y, ALC-Z and ALC-X formulations prepared using conventional or PFV process.

6 Lipid Re-Purification Reduces Adduct Formation



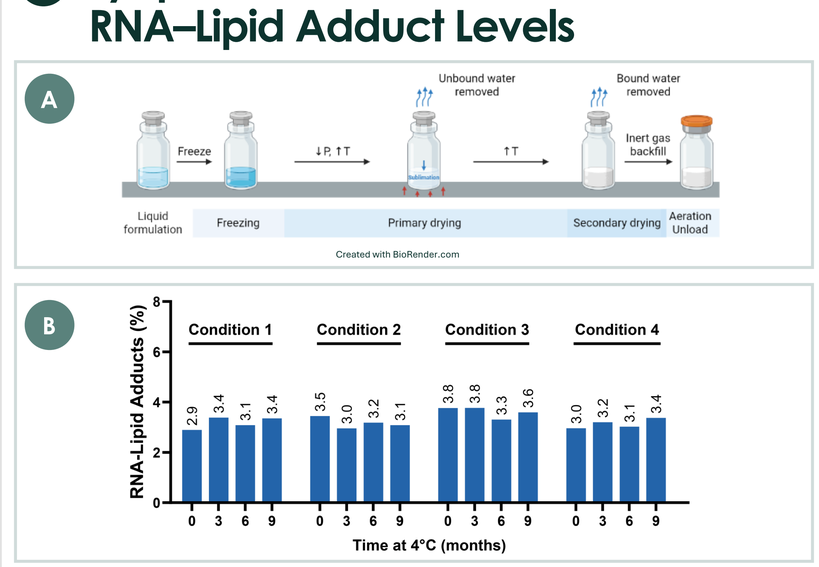
Lipid re-purification removes reactive impurities and reduces RNA–lipid adduct formation. (A) Ionizable lipid re-purification using orthogonal chromatography resins to remove reactive impurities. **(B, C)** N-oxide and aldehyde impurity levels and adduct formation with untreated or re-purified ALC-315® in **(B)** 1-day binary RNA-lipid mixtures or **(C)** LNP after 1 month of storage at −80°C or 4°C.

7 Tris Buffer Mitigates Aldehyde-Driven Adduct Formation



Tris buffer reduces RNA–lipid adduct formation under high-impurity conditions.
(A) Proposed reaction between aldehydes and Tris, forming a Tris–lipid adduct. **(B)** RNA–lipid adduct levels in LNP formulations with low or high impurity levels following 2 weeks of storage at room temperature in PBS or Tris buffer.

8 Lyophilization Maintains Low RNA–Lipid Adduct Levels



Lyophilized LNP formulations maintain stable RNA–lipid adduct levels during storage at 4°C.
(A) Schematic of the lyophilization process. **(B)** RNA–lipid adduct levels in LNP formulations prepared under four buffer conditions, stored as lyophilized powders at 4°C for 9 months, and reconstituted before analysis.

Summary

1. Aldehyde and N-oxide impurities in ionizable lipid raw materials drive RNA–lipid adduct formation, compromising *in vivo* protein expression.
2. Adduct-resistant ionizable lipids substantially reduce N-oxide–driven adduct formation during storage, even when challenged with N-oxide impurity.
3. Orthogonal chromatography removes reactive lipid impurities and reduces RNA–lipid adduct formation in both binary mixtures and formulated LNP.
4. PFV-based formulation process enables rapid RNA loading in minutes while reducing RNA–lipid adduct formation compared with conventional process.
5. Tris buffer reduces RNA–lipid adduct formation, particularly under high-impurity conditions, consistent with aldehyde scavenging.
6. Lyophilized LNP formulations maintain stable RNA–lipid adduct levels during long term storage at 4°C across four buffer conditions.

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References

1. Packer, M. et al. A novel mechanism for the loss of mRNA activity in lipid nanoparticle delivery systems. Nat Commun 12, 6777 (2021).
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